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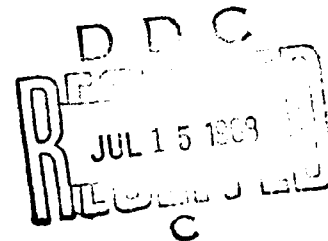Army Biological Defense Research Lab ltr dtd 29 Sep 1971

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TECHNICAL MANUSCRIPT 454

EFFECT OF CELL MOISTURE
ON THE THERMAL INACTIVATION RATE
OF BACTERIAL SPORES

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 454

EFFECT OF CELL MOISTURE ON THE THERMAL
INACTIVATION RATE OF BACTERIAL SPORES

Robert K. Hoffman

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Physical Defense Division
COMMODITY DEVELOPMENT AND ENGINEERING LABORATORY

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ABSTRACT

Thermal inactivation rates were determined for two strains of
Bacillus subtilis var. niger spores after equilibration to various
relative humidities (RH). In these tests, small thin stainless
steel squares were each inoculated with a drop of spore suspension
and equilibrated to 11, 33, or 85% RH. Following equilibration,
the squares were placed on a hot plate preheated to 108, 125, 136,
164, or 192 C for various exposure times, then assayed for sur-
viving organisms. The results revealed that spores of the A strain
of B. subtilis were least resistant if pre-equilibrated to 11% RH
and most resistant if pre-equilibrated to 85% RH. The same trend
was obtained at all temperatures except 192 C, at which no
difference was noted, probably because the rapid kill time approaches
the heat-up time of the stainless steel square. The B strain of
B. subtilis spores showed an opposite relative humidity effect; that
is, the cells pre-equilibrated to 11% RH were the most resistant.
Because the two strains of spores were grown on different media,
further studies were conducted at 136 C after subculturing the cells
on different media. When the B strain was subcultured on the A
strain medium, the pattern was reversed; the cells pre-equilibrated
to low relative humidity were then least resistant. Although it
was not possible to reverse these cells to the original pattern by
subculturing on the original B strain medium again, the pattern was
altered to the point that there was no significant difference in
heat resistance of these cells regardless of the pre-equilibration
relative humidity. The same result was obtained when the A strain
was grown on the B strain medium; that is, the thermal resistance
could not be reversed, but it was altered from the point where the
low - relative humidity equilibrated cells were least resistant
initially to the point where there was no significant difference
in any of the cells regardless of relative humidity used for
pre-equilibration.

The thermal resistance of spores seems to be dependent on (i)
the medium on which the spores are grown, (ii) the relative humidity
to which they are exposed before heating, and (iii) some genetic
characteristic of the cell.

I. INTRODUCTION

Interest in sterilization by low-temperature dry heat has increased within the past 7 or 8 years because of its consideration for sterilizing interplanetary spacecraft. Until recently, the effect that cell moisture plays on the thermal inactivation rate has been given little consideration. The most notable studies were conducted by Murrell and Scott,* who investigated the effect of cell moisture content on the rate of microbial death at dry-heat temperatures from 70 to 120 C. In the first of these studies, the microorganisms were dried at various relative humidities (RH), then heat-treated in closed vials at ambient, but obviously low, relative humidity. For all the bacterial spore species tested, pre-equilibration to 80 to 90% RH produced greatest resistance of spores to dry heat, but spores pre-equilibrated to greater or lesser relative humidities were less resistant. In the second study, the spores were pre-equilibrated and heated at the same relative humidity in closed vials. These results showed that spores equilibrated and tested at 20 to 40% RH were the most resistant to heat inactivation, but spores at relative humidities above or below this amount had lower heat resistance.

The present investigation was undertaken to determine the effect of cellular moisture content on the thermal inactivation of spores exposed to temperatures generally higher than those used by Murrell and Scott, i.e., 108 to 192 C.

II. MATERIALS AND METHODS

Bacillus subtilis var. niger has been used for more than 20 years as a test organism at Fort Detrick. During this time, cultures of the organism have been given to many other laboratories for test purposes. It has been an especially valuable agent in disinfection and sterilization studies, and many publications have appeared based on its use as a test organism. It has been implied that the numerous cultures are essentially the same; however, no comparative tests have been made to determine whether there are now differences in heat resistance or other properties in strains long separated.

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* Murrell, W.G.; Scott, W.J. 1957. Heat resistance of bacterial spores at various water activities. Nature 179:481-482.
  Murrell, W.G.; Scott, W.J. 1966. The heat resistance of bacterial spores at various water activities. J. Gen. Microbiol. 43:411-425.

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Thus, two strains of B. subtilis var. niger spores were used in this study. Both originated from single colony isolates from Fort Detrick strains. However, the one used in this laboratory (strain A) was grown on trypticase soy agar* (TSA) for 7 days, harvested, washed, and suspended in sterile distilled water; the other (strain B) was grown in a laboratory at the National Center for Urban and Industrial Health** on spore medium containing:

Glucose	0.25%
Casamino acids	0.25%
Yeast extract	0.25%
$MnSO_4 \cdot H_2O$	0.001%
$FeSO_4 \cdot 7H_2O$	0.0014%
Agar	1.5%

These cells were grown, harvested, and washed in a manner similar to that used for strain A. All spore suspensions were heat shocked at 60 C for 30 minutes before use to kill the less-resistant vegetative cells that might be present.

Thermal exposure was on a fabricated hot plate (Fig. 1) made of a 4 by 5 by 0.25 inch aluminum plate set on well-distributed heating elements and fixed in an asbestos base. The hot plate was suspended by non-heat-conductive material in a Transite box. A Transite hood was placed on top of the hot plate to assure more even heat distribution and to reduce heat radiation. Stainless steel squares 0.5 by 0.5 by 0.01 inch, placed on the aluminum plate, were used as the test surface. This technique was chosen because it provided a means of bringing the organisms up to the test temperature in a minimum time.

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* Baltimore Biological Laboratories, Baltimore, Md.
** Cincinnati, Ohio.

Figure 1. Fabricated Hot Plate for Thermal Exposure.

Each stainless steel square was contaminated with a single equal drop of aqueous suspension containing approximately 1×10^8 B. subtilis spores per milliliter. The inoculated squares were placed in desiccator jars containing saturated solutions of lithium chloride, magnesium chloride, or potassium chloride above which 11, 33, and 85% RH, respectively, is maintained at 25 C. The organisms were equilibrated for 48 to 72 hours.

After equilibration, 42 of the contaminated stainless steel squares were placed on the preheated hot plate; of these, 14 each were equilibrated to 11, 33, and 85% RH. No attempt was made to control the humidity surrounding the hot plate, but it was obviously low; e.g., even 100% RH air at 25 C will be reduced to 3% RH when heated to 108 C. After various exposure times, test squares were removed and immediately placed in sterile water blanks containing 0.01% non-ionic detergent and 0.3 to 0.4 g of coarse white sand. The detergent and sand facilitated the removal of spores from the stainless steel. Pour plates were prepared with tryptose agar to assay the surviving fraction of spores. All plates were incubated at 37 C for 48 hours before counting.

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Temperatures used in this study were 108, 125, 136, 164, and 192 C.
They were determined by the melting points of Tempilstik* markings on
stainless steel shims. A number of shims were marked with two Tempilstiks,
one that melts at 1 degree below the desired temperature and one that melts
1 to 2 degrees above that temperature, so that one Tempilstik sample
was melted and the other was not in all tests. To ensure that it did not
rise above that desired, the temperature also was monitored by thermocouples.
For that reason, the temperatures noted are plus or minus about 1 C. The
Tempilstik-marked shims were placed at corner and central locations on the
hot plate to assure that an even heat distribution was obtained. The air
temperature a few millimeters above the hot plate was only a few degrees
below the hot plate temperature; however, temperature fell rapidly above
this height.

Following the initial tests at all five temperatures, further tests
were conducted at 136 C to see whether the cause of the marked difference
in results with the two strains could be determined. First, both strains
of B. subtilis var. niger spores were subcultured on TSA and then harvested
and cleaned as before; both strains were then equilibrated to the same
three relative humidities used above, and the death rates were determined
at 136 C. Second, both strains were grown on the spore medium and tested
as before. Finally, a third test was run on both strains of spores that
had been grown on the spore medium in the second test and then grown on
TSA; these cells were harvested and tested as before.

All data for all tests represent the average of four samples obtained
from two samples in each of two tests.

III. RESULTS AND DISCUSSION

The death rates plotted on a semilogarithmic scale were all straight
line functions from which D values, or decimal reduction times, could be
readily calculated. Bar graphs showing the D values for each strain of
organism tested, at the three relative humidities and five temperatures,
are presented in Figures 2 and 3. The D values are expressed in seconds,
but a different time scale was necessary for each temperature because
of the great variation from one temperature to the next. Figure 2 shows
that strain A spores equilibrated to 11 and 85% RH were, respectively,
the least and the most resistant to thermal inactivation. This was true
for all temperatures except 192 C, where no difference was noted, probably
because the rapid death time at this temperature approaches the time
required to heat the stainless steel square surface; therefore, the
usefulness of the test procedure is limited to temperatures somewhat
below 192 C. Figure 3 shows the D values for strain B of B. subtilis var.

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* Tempil Corp., 132 W. 22nd Street, New York, N.Y.

niger spores equilibrated to the same three relative humidities and five temperatures. In this case, the effect of relative humidity on the organism was the reverse of that seen with strain A. In other words, the cells equilibrated to the low relative humidity were more resistant than those equilibrated to the high relative humidity at each temperature. Again, at 192 C, the death time was very short, and the differences in resistance among the three relative humidities are not significant.

Because the variation in results with the two strains may have resulted from the fact that they were grown on different media, the two strains were subcultured, first on TSA, then on the strain B spore medium, and then on TSA again, and they were tested after growth on each medium. Figure 4 shows the D values for strain A grown on the different media and tested only at 136 C. Regardless of which medium the cells were subcultured on, those equilibrated at the low relative humidity were always more susceptible to heat sterilization than were those equilibrated to the high relative humidity. Statistical analysis showed that the D values at 11 and 85% RH were not significantly different for strain A spores when grown on spore medium; however, the D values for 11 and 85% RH were significantly different whenever the cells were grown on TSA medium.

Figure 5 shows the D values for the B strain spores grown on the different media. Of great interest here is the reversal of the resistance at the various relative humidities. That is, B strain spores originally grown on spore medium were more resistant when equilibrated at low relative humidity than when equilibrated at high relative humidity, but the reverse was true when this strain was grown on TSA. Analysis showed that the D values for the low and high relative humidity tests were significantly different in both cases. Growing the cells on spore medium after growth on TSA did not give a pattern similar to that of the original B strain; however, low and high relative humidity D values were not significantly different. Growth on TSA did produce spores that had significantly different D values for the low and high relative humidity pre-equilibrations.

Both B. subtilis strains used in this study were initially isolated from single bacterial colonies. Why the D value pattern of the B strain was reversed when grown on TSA but failed to revert to the original pattern when subcultured on the spore agar is not clear. It appears that we are dealing with a genetic characteristic because the colonial color and formation did appear to change somewhat with successive transfers on the various media. This occurred even though the seed was obtained by removing a loopful of spores from 10 to 15 typical colonies on an agar surface each time the spores were grown. Close inspection of the data obtained with the A strain of B. subtilis spores and that obtained by Murrell and Scott* in their studies with Clostridium botulinum spores shows a similar trend of increased thermal resistance after equilibration to higher relative humidities. Both of these studies were performed without controlling the RH during the heating process.

* Murrell, W.G.; Scott, W.J. 1957. Heat resistance of bacterial spores at various water activities. Nature 179:481-482.
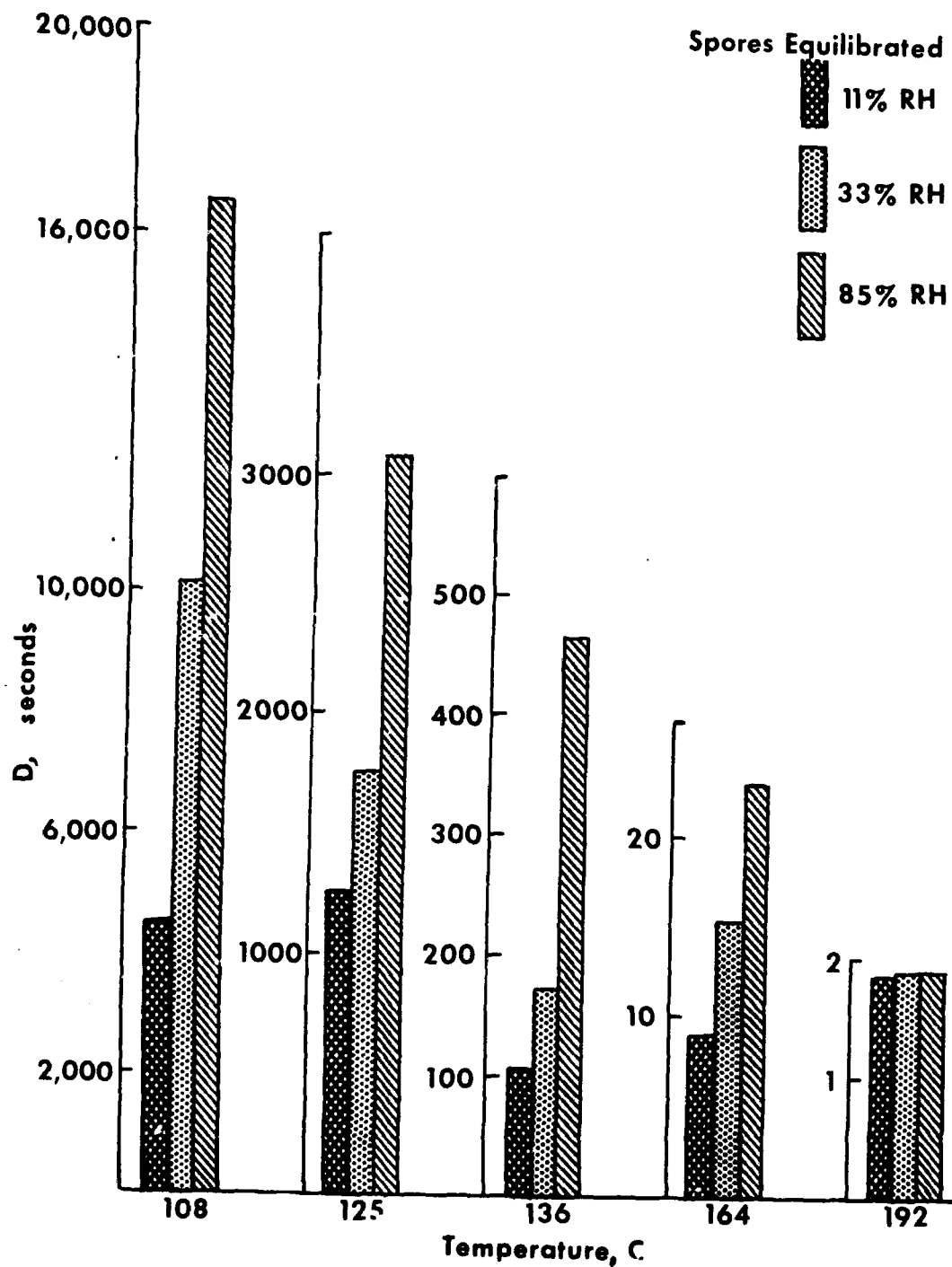
Figure 2. D Values for Strain A B. subtilis Spores Exposed
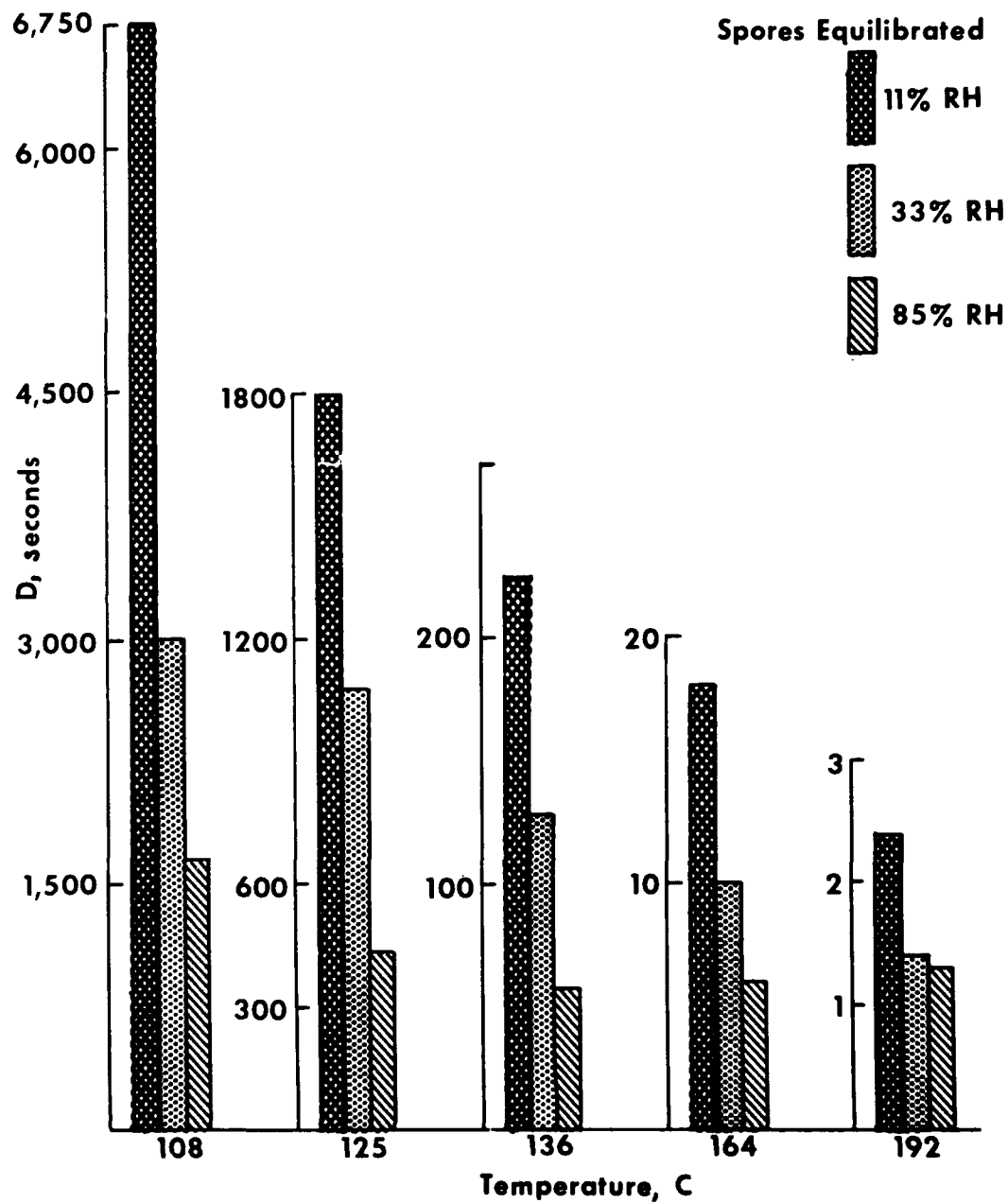to Five Temperatures after Equilibration to Three
Relative Humidities.

Figure 3. D Values for Strain B *B. subtilis* Spores Exposed
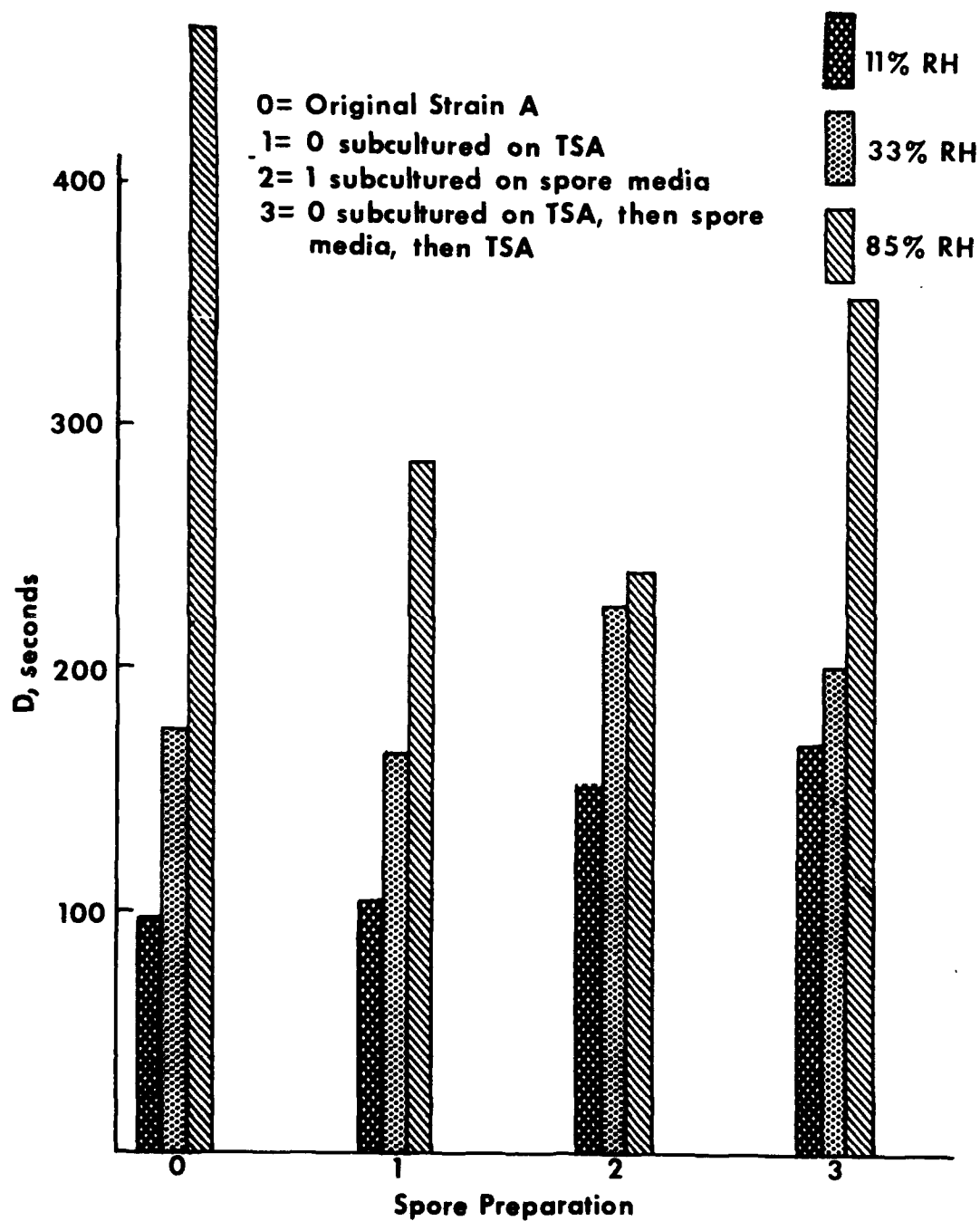to Five Temperatures after Equilibration to Three
Relative Humidities.

Figure 4. D Values for Strain A B. subtilis Spores Grown on
Various Media and Then Exposed to 136 C after
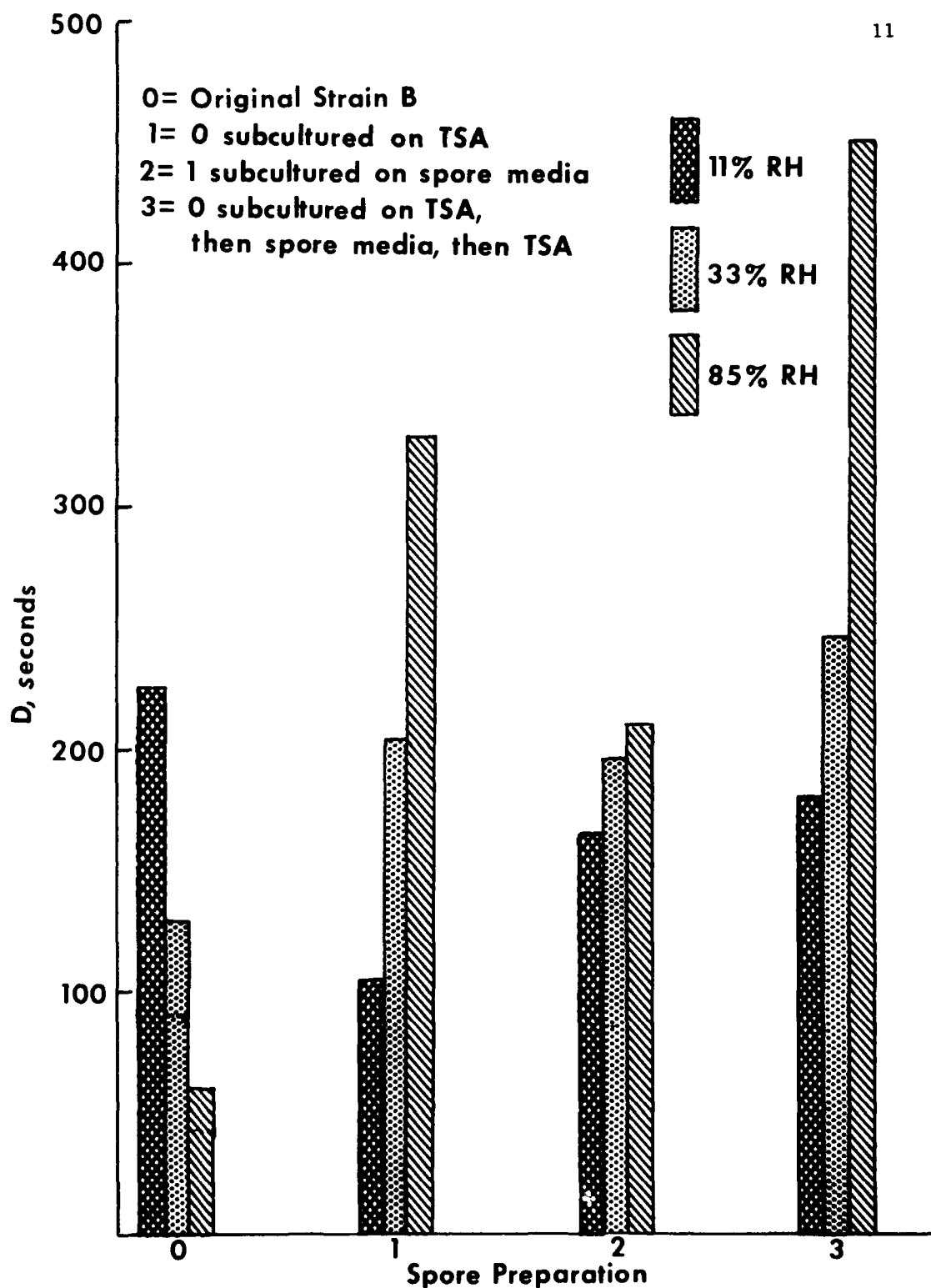Equilibration to Three Relative Humidities.

Figure 5. D Values for Strain B **B. subtilis** Spores Grown
on Various Media and Then Exposed to 136 C
after Equilibration to Three Relative Humidities.

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Three important facts stand out from this study: (i) broad generalities should not be made about the heat resistance of spores as a result of their preliminary exposure to any specific relative humidity; (ii) the medium on which the organisms are grown appears to have some bearing on the subsequent resistance of the cells to heat; (iii) the relative humidity to which the cells are exposed before heat sterilization plays an important role in their subsequent rate of kill.

The equilibration of the cell to various relative humidities does more than just vary the moisture content of the cell; it also causes a more "permanent" change in thermal resistance. Water freely moves in and out of the cell,* yet the D value pattern at the various relative humidities is the same for either strain of <u>B. subtilis</u> spores, whether tested at 108 or 164 C (Fig. 2 and 3).

If the amount of cell moisture is the primary factor in regulating the heat inactivation rate, one would expect to find no differences in D values for the cells equilibrated to 11, 33, and 85% RH at the lower temperature (e.g., 108 C). At this temperature, the D values are so high that most of the water molecules have ample time to evaporate from the cell, certainly long before sterilization is accomplished. Yet no deviation from linearity was observed in the log per cent survival vs. time plots from beginning to end. This indicates that the important factor in regulating the D values in the system used here is not the amount of moisture but where and how it is bound in the cell.

Spores heated in a dry atmosphere rapidly lose most of their moisture; therefore, the rate of their inactivation by heat appears to be controlled by a relatively few water molecules left in the cell. If one plots the D value as a function of the relative humidity for the three humidities used, the curves in general are not linear. The curves of Murrell and Scott likewise are not linear when plotted as a straight function of D value vs. relative humidity. This indicates that the rate of heat inactivation of the cell is not a simple function of the relative humidity.

At this time, there is no clear explanation of the mechanism by which the cell is inactivated by dry heat. It is evident that heat inactivation is a complicated mechanism that hinges on the medium on which the cell was initially grown, the presence or absence of water molecules in certain internal cell locations, and, probably, some genetic characteristic of the cell.

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* Hoffman, R.K. March 1968. Effect of bacterial cell moisture on the sporicidal activity of beta-propiolactone vapor, (Technical Manuscript 442). Physical Defense Division, Fort Detrick, Frederick, Maryland.

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13. ABSTRACT Thermal inactivation rates were determined for two strains of Bacillus subtilis var. niger spores after equilibration to various relative humidities (RH). In these tests, small thin stainless steel squares were each inoculated with a drop of spore suspension and equilibrated to 11, 33, or 85% RH. Following equilibration, the squares were placed on a hot plate preheated to 108, 125, 136, 164, or 192 C for various exposure times, then assayed for surviving organisms. The results revealed that spores of the A strain of B. subtilis were least resistant if pre-equilibrated to 11% RH and most resistant if pre-equilibrated to 85% RH. The same trend was obtained at all temperatures except 192 C, at which no difference was noted, probably because the rapid kill time approaches the heat-up time of the stainless steel square. The B strain of B. subtilis spores showed an opposite relative humidity effect; that is, the cells pre-equilibrated to 11% RH were the most resistant. Because the two strains of spores were grown on different media, further studies were conducted at 136 C after subculturing the cells on different media. When the B strain was subcultured on the A strain medium, the pattern was reversed; the cells pre-equilibrated to low relative humidity were then least resistant. Although it was not possible to reverse these cells to the original pattern by subculturing on the original B strain medium again, the pattern was altered to the point that there was no significant difference in heat resistance of these cells regardless of the pre-equilibration relative humidity. The same result was obtained when the A strain was grown on the B strain medium; that is, the thermal resistance could not be reversed, but it was altered from the point where the low - relative humidity equilibrated cells were least resistant initially to the point where there was no significant difference in any of the cells regardless of relative humidity used for pre-equilibration.

The thermal resistance of spores seems to be dependent on (i) the medium on which the spores are grown, (ii) the relative humidity to which they are exposed before heating, and (iii) some genetic characteristic of the cell.

14. Key Words
   *Bacteria
   *Spores
   *Bacillus subtilis var. niger
   Cell
   Moisture
   Inactivation
   Temperature
   Relative humidity
   Growth media
   Sterilization